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DNA adducts in fish following an oil spill exposure

Abstract On 12 December 1999, one third of the load of the Erika tanker, amounting to about 10,000 t crude oil flowed into sea waters close to the French Atlantic Coast. This oil contained polycyclic aromatic compounds (PAC) that are known to be genotoxic. Genotoxic effects induce DNA adducts formation, which can thus be used as pollution biomarkers. Here, we assessed the genotoxic impact of the "Erika" oil spill by DNA adducts detection in the liver of immature fishes (*Solea solea*) from four locations of the French Brittany coasts. Two months after the spill, a high amount of DNA adducts was found in samples from all locations, amounting to 92–290 DNA adduct per 10^9 nucleotides. Then total DNA adduct levels decreased to reach about 50 adducts per 10^9 nucleotides nine months after the spill. In vitro experiments using human cell cultures and fish liver microsomes evidence the genotoxicity of the Erika fuel. They also prove the formation of reactive species able to create DNA adducts. Furthermore, in vitro and in vivo DNA adducts fingerprints are similar, thus confirming that DNA adducts are a result of the oil spill.

Keywords DNA adduct · Erika oil spill · HepG2 cell · *Solea solea* · Fish microsomes

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Introduction

On 12 December 1999, the tanker "Erika" broke into two parts approximately 30 miles off the French coast and released about 10,000 t of fuel in the sea at "Point of Penmarc'h", South Finistère, France. Four hundred kilometers of Atlantic coast, from Morbihan to Vendée, were impacted between 23 December 1999 and February 2000 (see Fig. 1). Various analyses performed by independent institutes identified the spilled oil as fuel No. 6: CAS No. 68553-00-4 or "Bunker C" or fuel No. 2 in French nomenclature. In addition to paraffinic, cycloparaffinic, olefinic compounds, the "Erika" fuel oil contained a mixture of polycyclic aromatic hydrocarbons (PAH), polyclyclic heterocyclic carbons (HPH) including carbazoles, thiophenes and heavy metals (Boudet et al. 2000; IARC 1989; Mazeas and Budzinski 2002). Residuel "Erika" petroleum contamination has been detected on the feather of bird collected on Atlantic shore after the accident (Mazeas and Budzinski 2002) and also in sediments (Mazeas and Budzinski 2001). Cicollela (2000), also pinpointed the potential risks of the "Erika" fuel on the aquatic ecosystem exposed to the water soluble fraction of the fuel.

Bunker C is a complex mixture, which belongs to petroleum fuels classified by IARC in Group 2B, possibly carcinogenic to humans, which thus can react with DNA. Xenobiotic metabolization can lead to reactive compounds able to interact with DNA to form DNA adduct. The presence of a DNA adduct in a critical gene provides the potential for occurrence of a mutagenic event, resulting in subsequent alterations in gene expression and a loss of growth control (for a review see Poirier et al. 2000). The detection of DNA adducts is widely used as biomarker of aquatic contamination (Burgeot et al. 1996; Boillot et al. 1997; Ericson et al. 1998; Ericson and Larsson 2000; Harvey et al. 1997; Kurelec et al. 1989a,b; Lyons et al. 1999a,b, 2000; Stein et al. 1994; Stephensen et al. 2000; Varanasi et al. 1989).

Authors have demonstrated that DNA adduct in fish liver represent an exposure to genotoxic substance over a long period as compared to their measurement in blood which

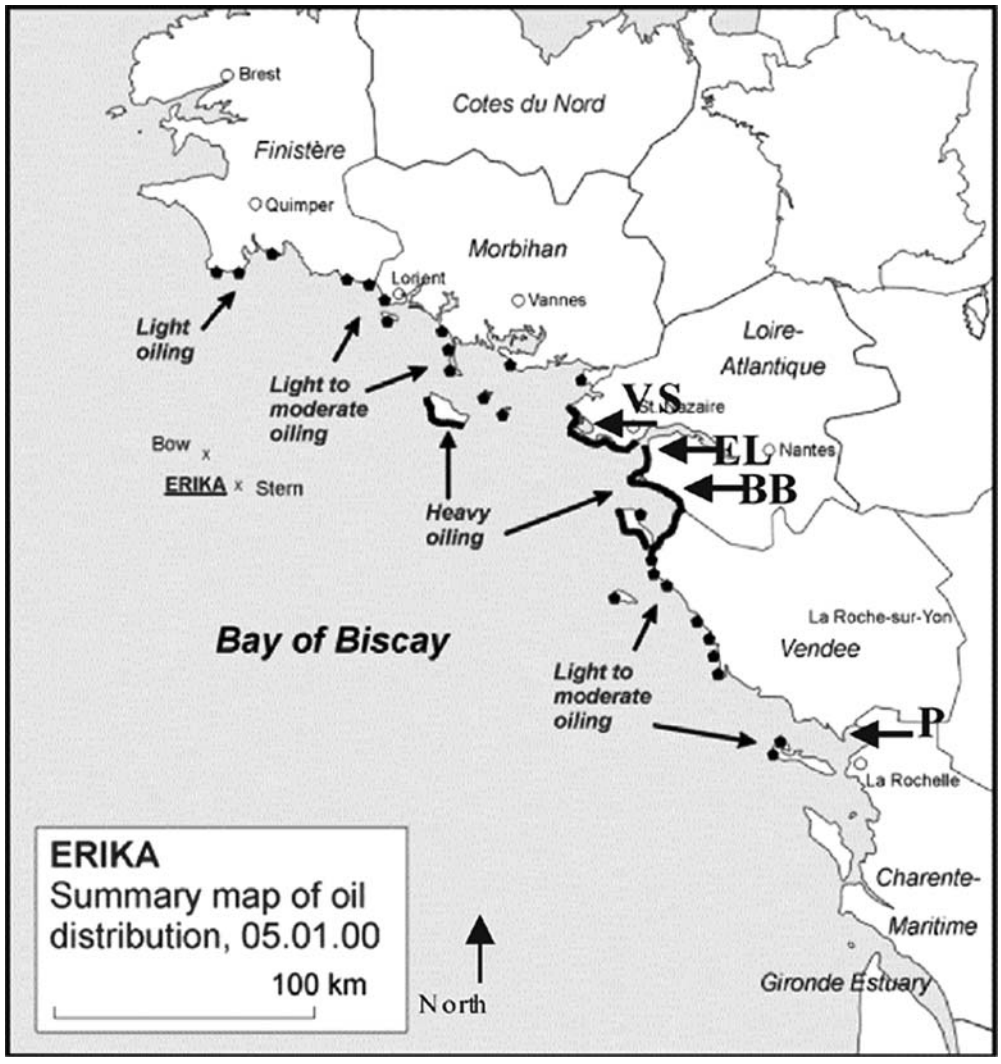

Fig. 1 Map showing the locations of spill. *Arrows* indicate the sampling locations from North to South of the impacted region. *VS: Vilaine* south; *EL*: Loire Estuary; *BB*: of Bourgneuf Bay, *P*: Pertuis. *Black points* represent the distribution of oil loaded on the coast on 5 January 2000 (ITOPF, International Tanker Owners Pollution Federation Limited)

represents a very recent exposure (Ericson et al. 1999; Telli-Karakok et al. 2001). Recently, Rice et al. (2000) used English Sole (*Pleuronectes vetulus*) to test the genotoxic impact of polluted sediment. Petrapiana et al. (2002) demonstrated that bottom dwelling flatfish, such as *Lepidhorombus boscii*, are sensitive species to evaluate histopathologic damages. Nine years after the *haven* oil spill (Ligurian Sea, Italy, 1991) they found positive response in liver of these flatfishes living in sites contaminated by hydrocarbons residues. Moreover, DNA adducts and the prevalence of degenerative hepatic lesions in aquatic species living in contaminated areas have been established (Baumann 1998; Ericson et al. 1998; Myers et al. 1998; Reichert et al. 1998; Van Schooten et al. 1995; Vincent et al. 1998).

To investigate whether fishes living in the vicinity of the "Erika" tanker oil spill suffered genotoxic damage, we analyzed the DNA adducts in liver of fish *Solea solea*, a bottom-dwelling species, at three different time periods: February, July, and September 2000. DNA adducts formation study of an Erika fuel extract, after metabolic activation, was investigated using hepatic human cell line (HepG2) and liver microsomes, extracted from fishes (*Solea solea*) exposed to the oil spill.

Experimental

Field sampling

Groups of 10 juvenile sole fishes (*Solea solea*) were collected at four locations along the French Brittany coasts (Fig. 1). These fishes live solitary in burrows into sandy and muddy bottoms and eat worms, molluscs and small crustaceans during the night. Livers were immediately excised, frozen and stored at -80°C prior to analysis. Two pools of five livers were analyzed at each location. Site 1 (Pertuis), was selected as potential control area, being about 110 km away from the other three sampling.

In-vitro incubation

Extraction of "Erika" fuel

Two types of extraction have been performed. *Extraction with DMSO*: Polycyclic aromatic hydrocarbons (PAH) contained in "Erika" fuel (100 mg) were extracted with DMSO (1 ml) under agitation (1 h) at room temperature.

This extract was used for microsome incubation (DMSO: dimethylsulfoxide). *Asphaltene precipitation of petroleum*: exposed to HepG2 cells. The maltene fraction was extracted as described by Mazeas and Budzinski (2001). Briefly, 500 µl of pentane were added to a 20 mg amount of fuel. The sample is slowly shaken for 10 min and the supernatant centrifugated for 3 min at 700 rpm.The extraction is repeated 20 times. The pooled collected fractions were evaporated to 500 µl under a stream of nitrogen.

Chemicals

Nicotinamide adenine dinucleotide phosphate reduced ($NADPH_2$), bovine serum albumin (BSA), aprotinine, phenylmethylsulfonylfluorid (PMSF) and arachidonic acid (AA) were obtained from Sigma: St. Quentin Fallavier, France.

Purification of microsomes and incubation

Livers from fishes were homogenised in a buffer solution (potassium chloride (KCl) 1.15%, 50 mM Na_2KPO_4, pH 7.4), with phenylmethylsulfonyl fluoride (10 µg ml^{-1}) and aprotinine (5 µg ml^{-1}). The buffer volume was three times the organ weight. After an initial centrifugation at 9000 × *g* for 20 min, the supernatant was taken and ultracentrifuged at 105,000 × *g* for 1 h. The pellets were homogenised in 1–2 ml of pH 7.4 buffer containing Na_2KPO_4 (50 mM), KCl (0.15 M), EDTA (1 mM), dithiothreitol (DTT) (1 mM), glycerol (20%). The mixture was centrifuged again at 105,000 × *g* for 1 h. Finally, microsomes were suspended in the same buffer to obtain a final concentration of about 10 mg of protein and stored at − 80°C prior to analysis. All steps of the isolation were carried out at 4°C.

After determination of the protein level in microsomes by Bradford's method (Bradford 1976), 0.5 mg of microsomal proteins were incubated in vitro in the presence of 70 µg DNA and "Erika" fuel extract (10 µl) in 500 µl (final volume) of Tris-HCl 50 mM, EDTA 1 mM, pH 7.4. The mixture was incubated at 37°C for 3 min before addition of co-substrate. For measurement of cytochrome P 450 (CYP)-dependent DNA adduct formation, $NADPH_2$ (10 µl, 10 mg ml^{-1}) was added. For cyclooxygenases (COX) and lipoxygenases (LOX) dependent activity, arachidonic acid (AA) (10 µl, 1 mg ml^{-1}) was added. The mixture was then incubated at 37°C for 45 min. Two controls were added: (1) incubation of DNA without microsomes and (2) incubation of microsomes alone. All incubations were performed in triplicate.

HePG2 cell culture

Chemicals

The growth culture media Eagle's minimum essential medium (D-MEM with glutamax, 4500 mg l^{-1} de D glucose, sodium pyruvate), phosphate-buffer saline (PBS) and Trypsin were obtained from Gibco (Cergy pontoise, France). Hepatic cell line (HePG2) were obtained from ATCC (American Type Culture Collection, Mannass, USA). Benzo[a]pyrene was purchased from Sigma.

Cell culture conditions

Hepatic cell line (HePG2) were cultured in 75 cm^2 flasks with 10 ml of culture medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, at 37°C under 5% CO_2 in sterile conditions. After trypsin digestion, the cells were resuspended in this medium to obtain 1106 cells per ml. At 4th to 5th day after seeding, the medium was replaced with fresh medium (5% foetal bovine, 1% penicillin/streptomycin) before treating cells with "Erika" fuel extract and BaP dissolved in DMSO. Pentane and DMSO never exceed 0.1% of total incubation volume. The cells were incubated for 24 h in presence of 10 µg ml^{-1} of "Erika" "fuel" extract and 25 µg ml^{-1} of B(a)P. For each treatment, three flasks were required. At the end of the treatment, cells of each flask are harvested in a total of 8 ml of PBS. The cells harvested are pooled and centrifuged at 700 rpm at 4°C and suspended in 700 µl of SET (NaCl: 0.1 M; EDTA: 20 mM; Tris–Hcl: 50 mM; pH 8). After treatment with sodium dodecyl sulfate (SDS, 10%) and potassium acetate (5 M), DNA is purified by phenol/chloroform extraction (Pfohl-Leszkowicz et al. 1991).

^{32}P-Postlabeling method of analysis of DNA adducts

Chemicals

Proteinase K, ribonucleases A and T1 (RNase A and RNase T1), spleen phosphodiesterase and micrococcal nuclease were purchased from Sigma (L'Isle d'Abeau, France); T4 polynucleotide kinase and [^{32}P-ATP], 370 Tbq $mmol^{-1}$ (5000 Ci $mmol^{-1}$) were from Amersham (Les Ullis, France); nuclease P1 from Boehringer (Manheim, Germany); rotiphenol from Rothsichel (Lauterbourg, France); cellulose MN 301 was from Macherey Nagel (Düren, Germany); the polyethyleneimine (PEI) was from Corcat (Virginia Chemicals, Portsmouth, VA, USA). The PEI-cellulose plates for thin layer chromatography (TLC) were made in the laboratory.

The ^{32}P-postlabeling procedure

For the ^{32}P-postlabeling method, samples were pooled in two duplicates. DNA were extracted and purified as described previously (Pfohl-Leszkowicz et al. 1991). The ^{32}P-postlabeling method is the one originally described by Reddy and Randerath (1986) with minor modifications. In brief, DNA (7 µg) was digested at 37°C for 4 h with micrococcal nuclease (183 mU) and spleen phosphodiesterase (12 mU) in a reaction mixture (total/volume 10 µl) con-

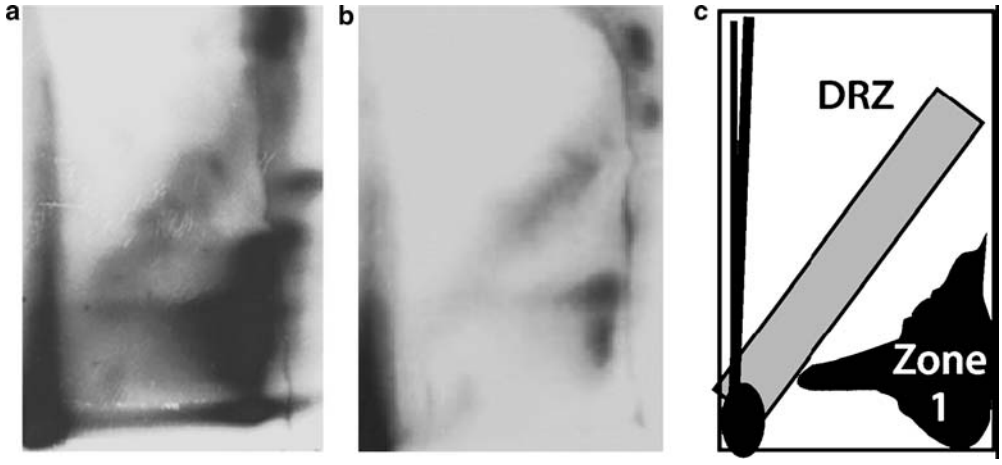

Fig. 2 Typical autoradiogram of adducts detected in the liver of *Solea solea* after the "Erika" oil spill at all locations. (**a**) in February (BB location); (**b**) in September (BB location); (**c**) Scheme and numbering of the bulky DNA adducts zones detected by the ^{32}P-postlabeling method

taining 20 mM sodium succinate and 10 mM $CaCl_2$, pH 6. Subsequently, adducted nucleotides are enriched. Digested DNA was treated with nuclease P1 (6 μg) at 37°C for 45 min before ^{32}P-postlabeling. Normal nucleotides, pyrophosphate and excess ATP were removed by chromatography on polyethyleneimine cellulose plates in 2.3 M NaH_2PO_4, pH 5.7 (D1) overnight. Origin areas containing labelled adducted nucleotides were cut out and transferred onto another polyethyleiminecellulose plate, which was run in urea 8.5 M, lithium formate 5.3 M, pH 3.5 (D2). Two further migrations (D3 and D4) were performed perpendicularly to D2. The solvent for D3 was lithium chloride 1 M, urea 8 M, Tris, HCl 0.5 M, pH 8, and the buffer D4 was 1.7 M NaH_2PO_4, pH 6. Autoradiography was carried out at −80°C for 24 or 48 h in the presence of an intensifying screen. The radioactivity of the spots is analyzed by a phosphoimager equipped with an Ambis software treatment system. In the analysis of each batch of liver DNA, a BaP modified standard obtained during the European Union collaborative study on ^{32}P-postlabeling validation method (Phillips et al. 2000) was used as positive control. A negative control was also analyzed each time. The ^{32}P-postlabeling assay is a highly sensitive method (limit of detection 1 adduct per 10^{10} nucleotides), in which the adducted nucleotides obtained by DNA digestion, were radioactively labelled (Phillips and Castegnaro 1999, 2000). The number of adduct is expressed in Relative Adduct Level (RAL)/10^9 nucleotides.

Results and discussion

Field study

The aim of this study was to investigate and follow the genotoxic impact of "Erika" fuel on marine environment. Part of "Erika" oil has been trapped by sediments and consequently could contaminate the animals living in sediments for a long time. Figure 2a presents a typical DNA adduct patterns detected in liver of fishes sampled at four locations along the French Brittany coast—Perthuis (P), Bay of Bourgneuf (BB), Loire estuary (EL) and Vilaine South (VS) in February 2000. Two different radioactive zones can be delimited on the autoradiogram (Fig. 2c): a diagonal radioactive zone (DRZ) and zone 1 (Z1). In February, DRZ and Z1 are observed in all samples, even in Perthuis location (P), which was expected to be a non contaminated area. Presence of DNA adduct in Perthuis location is not surprising. Indeed, ITOPF has published on 5 January a map showing the presence of "Erika" oil also in the region (Fig. 1). Each radioactive zone (DRZ and Z1) represents a lot of different individual DNA adducts, generated by the cross reactivity of genotoxic compounds with DNA. The DRZ is typical of a contamination by polycyclic aromatic hydrocarbons (PAH) (Ericson and Larsson 2000; Lyons et al. 1999a,b; Randerath et al. 1988; Schilderman et al. 1999). The highest DNA adduct levels (Fig. 3) are detected in February, two months after the accident in all areas. The DNA adduct levels reached 286 and 210 adducts/10^9 nucleotides, respectively in liver of fishes caught in BB and VS. In July, an important decrease of the total DNA adduct levels was observed in VS (76.4%) and EL (85.2%) but only a moderate decrease (27%) in BB. In Perthuis (P), the average of DNA adducts remained similar to that observed in February with a level of 160 adducts/10^9 nucleotides. In September, DNA adduct levels at all locations were very low (about 50 adducts/10^9 nucleotides) compared to those observed in February. The faster DNA adducts decrease

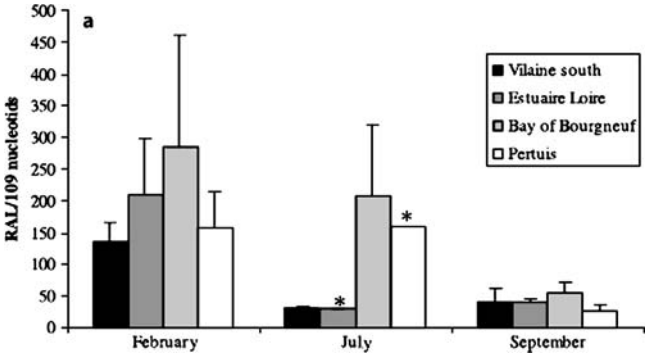

Fig. 3 Quantification of total DNA adducts detected in vivo in fish livers exposed to "Erika" oil spill. DNA adduct level expressed as number of adduct per 10^9 nucleotides

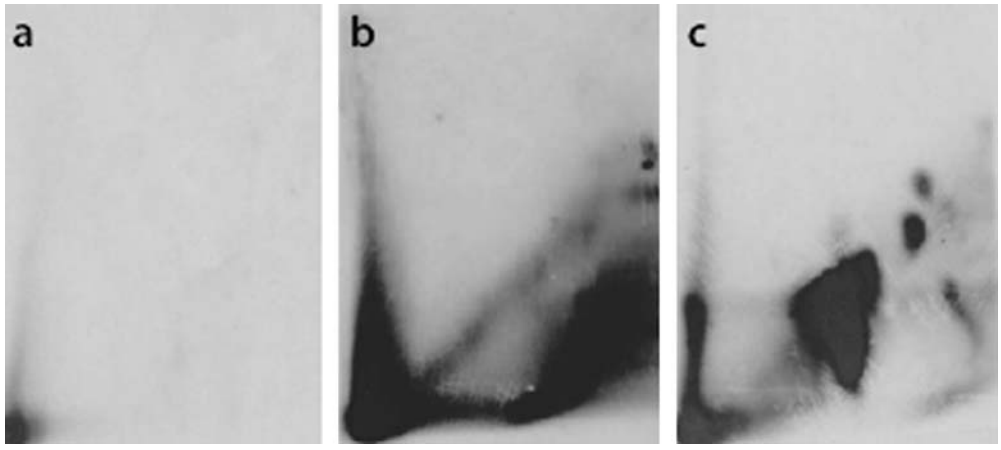

Fig. 4 DNA adduct pattern after incubation of HePG2 cells for 24 h. (**a**) Control; (**b**) cells incubated with "Erika" fuel extract; C) cells incubated with benzo[a]pyrene

in EL and VS, could be explained either by precence in samples of migrated juvenile (Amara et al. 2000) coming from non polluted sites, or/and pollution dilution by input of new river sediments. In contrast, the long persistence of DNA adduct in south sites (BB and P) is probably due to the contamination of the sediments. Observations made by C.E.D.R.E. indicate that BB location has been strongly and regularly impacted by oil slick, at least until April 2000 (www.le-cedre.fr).

These results are in accordance with those from Lyons et al. (1997) and Harvey et al. (1999) who analyzed the genotoxic impact of the *Sea Empress* crude oil on fish tissues (*Lipophorys pholis* and on *Limanda limanda*), as well as on invertebrate species (*Halichondira panicea* and *Mytilus edulis*). DNA adduct patterns of fish liver exhibited a typical diagonal zone (DRZ). 12 to 17 months after the spill, DNA adducts persisted in vertebrates but not in invertebrates species. It has also been found that DNA adducts in fish liver are very persistent (Ericson et al. 1999; Stein et al. 1994; Varanasi et al. 1989 in Ericson and Larsson 2000). French et al. (1996) observed a steady increase in DNA adduct levels during a chronic exposure of English sole (*Pleuronectes vetulus*) to PAH-contaminated sediment for 5 weeks which were very persistent even after a depuration period. In the same way, Aas et al. (2000), have also observed that hepatic DNA adduct formed in Atlantic cod (*Gadus morhua*) appeared 3 days after exposure to low concentration of crude oil and increased steadily during the 30 days following exposure. Over 60% of the DNA adducts remained after 60 days. Our first sampling was about 2 months after the accident, falling between these two time points. The data from February sampling present thus the situation between the maximum impact on DNA formation and its reduction by 40%. Our results demonstrated that the "Erika" fuel has a genotoxic impact on fish liver.

Genotoxic impact of "Erika" fuel on hepatocytes (human HePG2 cell)

We have no basis for comparison with the situation before the accident. To confirm that the genotoxic effect observed in fish liver is really related to "Erika" petroleum, HePG2 cells (human hepatocyte) were incubated for 24 h in presence of the "Erika" fuel extract or benzo(a)pyrene used as a positif indicator of the cells to biotransform PAH into genotoxic compounds (Fig. 4). Incubation of HePG2 cells with the "Erika" fuel extract lead to the formation of two radioactive zones (Fig. 4B). The total levels of DNA adduct reached 258 adducts/10^9 nucleotides. The pattern is similar to those obtained in Sole exposed to "Erika" oil in marine environment These data indicates that human hepatocyte biotransformed "Erika" fuel into genotoxic metabolites similarly to hepatic cell of fishes and confirmed that the adducts observed in field study are related to the contamination of sediment by the "Erika" fuel.

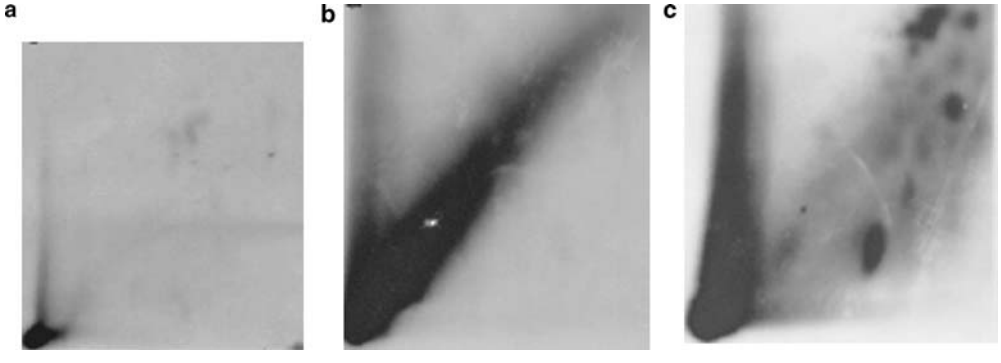

Fig. 5 DNA adduct pattern datected after in-vitro incubation of "Erika" fuel in presence of fish liver microsomes and DNA. (**a**) controls (Microsome + "Erika" fuel; b microsome alone; c microsome + DNA); (**b**) microsomes + "Erika" fuel extract + DNA + NADPH2; (**c**) microsomes + "Erika" fuel extract + DNA + AA

In-vitro biotransformation of "Erika" petroleum by fisher liver microsomes

To confirm that fish liver is able to biotransform the "Erika" fuel, fish liver microsomes were incubated with an Erika fuel extract and DNA. Two different co-substrates have been used to investigate two metabolic pathways: $NADPH_2$, needed for cytochrome P450 activity (CYP) and arachidonic acid (AA), needed for cyclooxygenase (COX) and lipoxygenase (LOX) activities. This ability of the microsomal fraction to transform PAH into species able to form DNA adducts has been previously reported in fish liver microsomes incubated with Benzo(a)Pyrene (Peters et al. 2002). In our study, a diagonal radioactive zone (DRZ) is observed when the "Erika" fuel extract is incubated in presence of microsomes, DNA and NADPH2 (Fig. 5B). These results prove the ability of liver fishes to biotransform "Erika" fuel extract into genotoxic compounds, able to form DNA adducts after activation by cytochrome P450. The DRZ observed in the in vivo pattern is here confirmed and result from a metabolic activation.

Several individual DNA-adducts are observed when the extract is incubated in presence of microsomes, DNA and AA (Fig. 5C). In our laboratory, we have previously demonstrated that depending of the PACs, genotoxic compounds are generated during biotransformation by CYP, COX and LOX (Genevois et al. 1998). Biotransformation of the components of the "Erika" fuel extract in presence of AA by COX and/or LOX induces the formation of several DNA adducts. Those have the same chromatographic properties as DNA adducts in the Z1 observed in vivo. These results demonstrate that "Erika" fuel is genotoxic after metabolic activation by cytochrome P 450, cylooxygenase and lipoxygenase.

Conclusion

Despite the fact that we have no comparison with the situation prior to the "Erika" oil spill, these results demonstrate a genotoxic event to the liver of the fishes living in the impacted zone. Altogether, our data confirm the interest of the of DNA adduct analysis in fish liver to follow the impact of a pollution by polycyclic aromatic hydrocarbons. We have confirmed the Erika fuel genotoxicity by in-vitro experiments and also that DNA adducts pattern observed in vivo is due to the metabolization, leading to genotoxic compounds and DNA adducts. Further studies are necessary to evaluate: (1) the metabolic pathway leading to the formation of genotoxic metabolites able to form DNA adducts, (2) the implication of the inflammatory processes on the genotoxicity observed.

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